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GRANT NUMBER DAMD17-96-1-6099

TITLE: Characterization of an In Vitro Human Breast Epithelial Organoid System

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REPORT DATE: August 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

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19971208 023

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REPORT DOCUMENTATION PAGE

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OMB No. 0704-0188

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1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE August 1997	3. REPORT TYPE AND DATES COVERED Annual (1 Aug 96 - 31 Jul 97)	
4. TITLE AND SUBTITLE Characterization of an In Vitro Human Breast Epithelial Organoid System			5. FUNDING NUMBERS DAMD17-96-1-6099	
6. AUTHOR(S) Chia-Cheng Chang, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Michigan State University East Lansing, MI 48824-1046			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Commander U.S. Army Medical Research and Materiel Command Fort Detrick, Frederick, Maryland 21702-5012			10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200) We have studied the expression of estrogen receptor (ER) in two types of normal human breast epithelial cells (HBEC) and their SV40 transformed cell lines. The results show that Type I HBEC expressed a variant ER (~48kd) by Western blot analysis. This variant ER contains a deletion in the DNA binding domain (exon 2) as revealed by RT-PCR analysis. In contrast, Type II normal HBEC are ER-negative. SV40 transformed Type I and Type II HBEC also expressed this variant ER. Tumors formed in athymic nude mice by <i>in vitro</i> transformed Type I cell lines, however, expressed a high level of wild type ER. Similar differential ER mRNA splicing was observed between these cells grown on plastic and in Matrigel. In addition, we have shown that: 1) Type I HBEC form budding/ductal and acini structures whereas Type II HBEC form hollow spheres and elongated cell mass on Matrigel; and 2) Type I HBEC exhibit telomerase activity which was continuously maintained during neoplastic transformation. These findings implicate Type I HBEC with stem cell characteristics as target cells for breast carcinogenesis and suggest a potential strategy to control the growth of estrogen-dependent tumor by modulating ER mRNA splicing.				
14. SUBJECT TERMS Breast Cancer, Estrogen Receptor, Alternative Splicing, Stem Cells, Telomerase, Breast Epithelial Organoid			15. NUMBER OF PAGES 27	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

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Chia-Cheng Chang Aug. 25, 1997
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INTRODUCTION

We have previously developed a culture method to grow two morphologically distinguishable types of normal human breast epithelial cells (HBEC) from reduction mammoplasty (1). These two types of cells are substantially different in phenotypes in many categories (1-3): 1) Type I HBEC are deficient in gap junctional intercellular communication (GJIC) whereas Type II HBEC are proficient in GJIC; 2) Type I cells express antigenic markers of luminal epithelial cells (i.e., epithelial membrane antigen and keratin 18) while Type II cells express basal epithelial cell markers (i.e., α -6 integrin and keratin 14); 3) Type I cells are more susceptible to neoplastic transformation by Simian Virus 40 (SV40) large T-antigen to acquire the ability to grow in soft agar (anchorage independent) and to become immortal; 4) Type I cells express an estrogen receptor (ER) whereas Type II cells are ER-negative; and 5) Type I cells but not Type II cells show stem cell characteristics (i.e., the ability of Type I cells to differentiate into Type II cells and the unique ability of Type I cells to form budding/ductal structures on Matrigel). Since breast cancers are very likely to be derived from stem cells and ER-positive HBEC, the major objectives of this project are to develop an *in vitro* organoid system using Type I and Type II HBEC to analyze factors effecting normal and abnormal growth and differentiation of human mammary gland and to characterize the structure and function of estrogen receptors expressed in normal and *in vitro* neoplastically transformed cell lines.

The specific objectives for the past year as listed in the Statement of Work are:

Task 1 (months 1-12): Characterization of budding and ductal structures formed by normal HBEC on Matrigel.

Task 3 (months 7-24): Comparison of ER expression in two types of normal HBEC grown on plastic and in Matrigel.

Task 4 (months 1-12): ER expression in SV40 large T-antigen transformed HBEC.

In addition, it has been reported that tumor cells expressed telomerase that maintain telomere length and normal cells in general lack the telomerase activity (4). However, recently, it has been shown that telomerase activity can be found in cells from human epidermal basal layer where stem cells are located (5). Since we have the putative human breast epithelial stem cells, we have examined telomerase activity in the two types of HBEC and their SV40 transformed cell lines during different stages of lifespan extension immortalization.

BODY

A. Expression of Estrogen Receptor in SV40 Large T-antigen

Transformed Human Breast Epithelial Cells

Rationale

Estrogen receptor plays an important role in the pathogenesis and maintenance of breast cancer. In breast cancer patients, about two-thirds of tumors are ER-positive (6); 50% of these ER-positive tumors are estrogen-dependent and respond to endocrine therapy (7). The origin of ER expression, however, is not known. The human mammary gland contains a small but distinct population of ER-positive cells, comprising ~7% of the total epithelial cell population from all biopsies (8). Normal HBEC grown in the commonly used media, MCDB 170 and DFCI-1, including the commercially available normal HBEC (Clonetics), exhibited basal epithelial, but not luminal epithelial cell characteristics. These cells have not been shown to express the estrogen receptor. Since our Type I HBEC possess stem cell and luminal epithelial cell characteristics and are more susceptible to neoplastic transformation, it is important to determine whether these cells and their SV40 transformed cell lines expressed the ER.

Methods and Procedure

The expression of ER was studied by Western blot analysis and immunostaining using anti-ER antibodies which recognize either the N-terminal or the C-terminal portion of the ER. These studies would reveal the molecular weight and domains of the expressed ER. The structure and the

presence of DNA-binding domain of the expressed ER were studied by RT-PCR and the ER-ERE (estrogen responsive element) binding assay respectively. The primers for PCR are sequences surrounding the border between exons 7 and 8 of the ER (9) and sequences encompassing exon 2 of the ER (10).

Results and Discussion

The major finding of this study is that an ER was expressed in all four of the HBEC examined while their Type II HBEC counterparts did not express any ER. Unexpectedly, ER was expressed in all SV40 transformed Type II HBEC as well as in Type I HBEC. The ER expressed in these cells, grown in culture on plastic, however, is not the wild type ER. It is a variant ER with smaller molecular weight (~48 kd) than the wild type ER (~66 kd). Furthermore, the ER was found to be deleted in the N-terminal DNA-binding domain as shown by studies with RT-PCR and immunostaining using anti-ER antibodies recognizing either the N- or C-terminal region and the ER-ERE binding assay. The expression of this variant ER appears to be an *in vitro* phenomenon, since *in vitro* transformed tumorigenic Type I HBEC expressed a high level of wild type ER in tumors formed in athymic nude mice. That the *in vivo* expressed wild type ER is functional is indicated by the expression of progesterone receptor which is inducible by the functional ER. When these tumors were grown as cell culture on plastic again, they expressed the variant ER. Thus, there appears to be a differential ER mRNA splicing between the *in vitro* and *in vivo* milieu.

The expression of ER in our Type I HBEC and SV40 transformed Type I and Type II HBEC is unambiguous since it has been observed by 3 different methods (i.e., Western blot analysis, immunostaining and RT-PCR). The expression of the variant ER in SV40 transformed Type II HBEC is unexpected. The mechanism for its expression is not clear. One possible mechanism is that the expression of large T-antigen induced the expression of ER. We have tested the hypothesis by transfecting the ER-negative MDA-MB-231 breast adenoma cells with SV40. The MDA-MB-231 cells expressing the large T-antigen resulted from SV40 transfection, however, remained ER-negative. Alternatively, in the Type II HBEC population, there might exist a small population of ER-positive transitional cells, newly differentiated from Type I cells, which were the target cells for SV40 transformation. Except for ER-expression, the phenotypes of SV40 transformed Type II HBEC are substantially different from that of SV40 transformed Type I cells (1). Therefore, these hypothetical transitional cells are quite different from the Type I HBEC.

This study of the ER expression in SV40 transformed HBEC (Task 4) has been completed. A paper reporting the results of this study was published (3). This is an extension of a previous study, supported by National Institute of Environmental Health Sciences, which aims at identifying ER-positive normal HBEC.

B. Comparison of ER Expression in Two Types of Normal HBEC Grown on Plastic Surface and in Matrigel

Rationale

As shown in the preceding study, *in vitro* transformed tumorigenic Type I HBEC expressed the wild type ER in tumors formed by these cells, yet the same cells expressed the variant ER when they were cultured *in vitro* on plastic surface. The mechanism underlying this differential expression is not known. It could be due to the extracellular matrix present in the tissue. If Matrigel can be shown to mimic the *in vivo* condition in inducing the expression of wild type ER, it could be used as a surrogate for animal to study the mechanism of alternative splicing of ER mRNA in comparison with the *in vitro* condition.

Methods and Procedure

In our preliminary study, two tumorigenic cell lines, M13SV1R2N1 and M13SV1R2N8 (derived from Type I HBEC after sequential treatment with SV40, x-rays and neu oncogene), were used. These cells were grown on plastic surface or embedded in Matrigel (Collaborative Biomedical Products, Becton Dickinson). The cells on plastic can be harvested for proteins with lysis solution. The cells in Matrigel after growing for different times were harvested using Matrisperse (Collaborative Biomedical Products) to remove the Matrigel before they were lysed for proteins. Protein extracts were also obtained from tumor tissues formed by these cells when they were dissected from animal or

after the dissected tissues were cultured on top of Matrigel for more than two weeks. The expression of ER was then studied by Western blot analysis using an anti-ER antibody (Ab-1, Calbiochem.) recognizing the C-terminal region of the ER.

Results and Discussion

As shown in Fig. 1A, we were able to repeat the previous results that tumorigenic Type I HBEC (M13SV1R2N1, N8) grown on plastic (CP) expressed only the variant ER (~48 kd), whereas the same cells expressed the wild type ER (~66 kd) and a different variant ER (~51 kd) when they formed tumors in male or female nude mice (TT). The expression of these ER by tumor cells can be maintained for more than two weeks when the tumor tissues were cultured on top of Matrigel but immersed under the serum-free MSU-1 medium (1) (TM). The result shown in Fig. 1B further indicates that these cells originally grown on plastic surface expressed the same wild type and variant ER after they were embedded in Matrigel (CM) for 4 days.

These results clearly demonstrate that Matrigel can mimic the *in vivo* condition to express the wild type ER. The system can be used to analyze what components in Matrigel are important for the expression of wild type ER. In follow-up experiments, we will determine if the expressed wild type ER on Matrigel are functional by measuring cell growth in medium with or without 17- β estradiol. We also will determine if normal Type I HBEC behave similarly.

C. Characterization of Budding and Ductal Structures formed by Normal HBEC on Matrigel

Rationale

We have previously shown that Type II HBEC formed hollow spheres while Type I HBEC formed acini and budding/ductal structures on Matrigel. The latter provides strong evidence that Type I HBEC contain both stem cell and luminal epithelial cells. The organoid system, when characterized, may be used as an *in vitro* model to study factors that affect normal and abnormal growth and differentiation of human mammary gland.

Methods and Procedure

Three areas of characterizations are to be performed: 1) To determine if different HBEC cultures are equally capable of forming these organoid structures; 2) What antigenic markers of breast epithelial cells are expressed in these structures; and 3) The three-dimensional structure as revealed by histological studies. These studies require the use of multiple HBEC cultures, immunostaining and histological staining of sectioned organoid structures. In this study, we had two technical developments: 1) Direct immunostaining of organoid on Matrigel; and 2) Using different vital stains (PKH-26, Sigma; CMFDA, Molecular Probes) to prelabel different types of cells to locate their roles in organoid formation.

Results and Discussion

Using two different HBEC cultures, we were able to repeat previous results showing that Type II cells formed hollow spheres and that Type I cells formed acini structures. A mixture of Type I and Type II cells formed budding and ductal structures. In addition, we also observed that Type II cells formed elongated cell mass. We also discovered that cells or organoid can be immunostained directly on Matrigel without showing the background staining of Matrigel. Using this method, we have observed that acini formed by Type I cells expressed the luminal epithelial marker, keratin 18 and that hollow spheres and elongated structures expressed the basal epithelial marker, keratin 14. The budding structure also expressed keratin 18. In mixed cell culture using vital stain prelabelled cells, we found that hollow spheres only formed by Type II cells and that budding structures were formed by Type I cells. Due to late recruitment of a research associate and extra effort needed to finish other projects, we have not done the histological study of organoids formed on Matrigel.

In these experiments, the structures of acini and hollow spheres can be consistently obtained by Type I and Type II cells respectively. The frequency of a mixture of Type I and Type II cells to form budding and ductal structures, however, varies among experiments. We suspect that it could be due to different ratios of Type I and Type II cells. This will be examined in future experiments. We have noted that the structure of our hollow spheres formed by Type II cells is similar to the squamous metaplasia formed by rat mammary

epithelial organoids cultured in Matrigel (11). We will conduct histological studies of our hollow spheres to determine if they have squamous metaplasia characteristics.

D. Telomerase Activity in a Normal Human HBEC Type with Stem Cell Characteristics and Its SV40 transformed Cell Lines

Rationale

Telomerase activity has been found in most cancer cells and in many immortal cell lines, as well as in germ line cells. However, it is generally undetectable in normal human somatic cells. Progressive telomere shortening and reactivation of telomerase activity have been considered as the key mechanisms respectively for cellular senescence and immortalization. However, some tissues with cell renewal ability, such as basal cells of the epidermis in human skin, have been reported to express telomerase activity (5). Since Type I HBEC possess stem cell characteristics, we would like to know if these cells and SV40 transformed cell lines derived from them express the telomerase activity. The result has implication concerning the origin of telomerase activity in tumor cells, i.e., reactivation vs. continuous expression from parental normal stem cells.

Methods and Procedure

Normal Type I and type II HBEC (HME15) and their SV40 transformed cells at different stages of lifespan extension and immortalization were assayed by the TRAP (4) to determine the expression of telomerase activity.

Results and Discussion

Both Type I and Type II HBEC were found to express telomerase activity at early passage. After prolonged culture, Type I cells retained whereas Type II cells lost the telomerase activity. Type I cells at different stages of SV40 induced lifespan extension and immortalization were found to express telomerase. Thus, the results support the persistent expression of telomerase in SV40 transformed Type I HBEC instead of reactivation of telomerase from telomerase non-expressing cells. These results have been presented at the 1997 AACR (Am. Assoc. Cancer Res.) annual meeting (Proc. Am. Assoc. Cancer Res. 38:504, 1997).

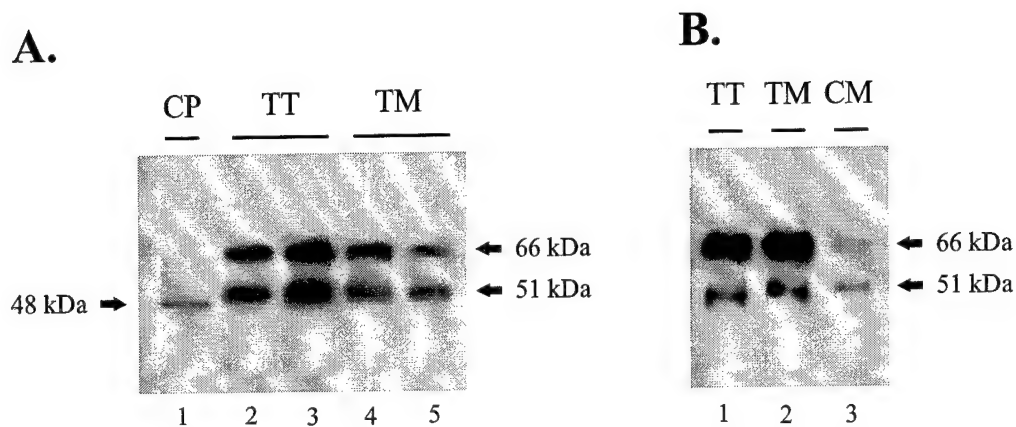


Figure 1. Western blot analysis of the estrogen receptor gene expression. The tumorigenic cell line derived from HBEC cells, M13SV1R2-N8, was harvested under different conditions: **CP**, cell-culture on plastic; **TT**, tumor tissues developed in nude mice; **TM**, tumor tissues removed from nude mice, then maintained on Matrigel for two weeks; **CM**, cells grown on plastic, then trypsinized as single cells and embedded in Matrigel for 4 days. Tumors were developed either in male nude mice (lanes A-2, A-4, B-1, B-2), or in female nude mice (lanes A-3 and A-5). Both Fig. 1A and 1B were assayed by Western blot using an anti-ER antibody from Calbiochem. Ab-1.

CONCLUSION

From the study of ER expression in normal HBEC, we found that Type I but not Type II cells expressed the ER. SV40 transformed HBEC also express the ER. The ER expressed in these cells, cultured on plastic surface, however, were found to be a variant ER deleting the DNA binding domain. The expression of this variant ER *in vitro* may be an indication that these cells may express the wild type ER *in vivo* since tumorigenic Type I cells expressed the wild type ER when they formed tumors in nude mice.

The implication of the results from this study are two-fold. First, the expression of ER in Type I HBEC provides additional similarity between breast cancer cells and Type I HBEC. As mentioned previously, the origin of the ER-positive tumors is not known. One possible origin is that they were derived from ER-positive normal HBEC stem cells similar to our Type I HBEC. During the neoplastic transformation, many of the parental target cell phenotypes are largely preserved. Those include deficiency in gap junctional intercellular communication, expression of luminal epithelial cell markers (1), expression of telomerase and ER (this study). Our study also indicates that Type I HBEC are more susceptible to neoplastic transformation by an oncogenic (SV40) stimulus (i.e., to become immortal and capable of anchorage independent growth) (1). The phenotypic similarity between Type I HBEC and breast cancer cells mentioned above are consistent with the notion of oncogeny as blocked or partially blocked ontogeny (12). Second, our results indicate that there is a differential splicing in ER mRNA under *in vitro* and

in vivo conditions and suggest a potential strategy to control the growth of estrogen-dependent human breast tumors, i.e., the modulation of the tissue environment in order to facilitate the alternative splicing that results in the expression of non-functional ER.

We also found that extracellular matrix may be important for the expression of wild type ER as we found that Matrigel may mimic the *in vivo* tissue condition to induce the expression of wild type ER. This system may further be used to identify factors regulating the ER expression.

This study provides further evidence that Type I HBEC are breast epithelial stem cells. Previously, we have shown that Type I cells may be induced by cyclic AMP enhancing agents to differentiate into Type II cells (1). In this study, we showed that Type I cells have the ability to form budding/ductal structure on Matrigel and to express the telomerase activity. Together with ER expression and evidence that they are more susceptible to neoplastic transformation, Type I HBEC appears to be the main target cells for breast carcinogenesis.

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APPENDICES

Reprint of a published paper:

K.S. Kang, I. Morita, A. Cruz, Y.J. Jeon, J.E. Trosko and C.C. Chang. Expression of estrogen receptors in a normal human breast epithelial cell type with luminal and stem cell characteristics and its neoplastically transformed cell lines.

Carcinogenesis **18**:251-257, 1997.

Expression of estrogen receptors in a normal human breast epithelial cell type with luminal and stem cell characteristics and its neoplastically transformed cell lines

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Although approximately two-thirds of breast cancers are estrogen receptor (ER)-positive, only a small proportion of epithelial cells in the mammary gland express the ER. The origin of the ER-positive breast cancers is unknown. Recently, we have developed a culture method to grow two morphologically and antigenically distinguishable types of normal human breast epithelial cells (HBEC) derived from reduction mammoplasty. In this report, we studied the expression of ER in these two types of cells and their transformed cell lines. The results indicate that Type I HBEC with luminal and stem cell characteristics expressed a variant ER (~48 kd) by Western blot analysis. This variant ER contains a deletion in the DNA binding domain (exon 2) as revealed by RT-PCR analysis. The lack of the DNA-binding domain of the variant ER was also confirmed by the ER-estrogen responsive element binding assay, as well as by the immunofluorescence staining of the ER using anti-ER antibodies which recognize either the C-terminal or N-terminal region. In contrast, Type II HBEC with basal epithelial phenotype are ER-negative. Simian virus 40 (SV40) transformed Type I and Type II HBEC lines also expressed the variant ER. Tumors formed in athymic nude mice by *in vitro* transformed tumorigenic Type I cell lines, however, expressed a high level of wild type ER which was undetectable in these cells grown *in vitro* before and after tumor formation. Thus, there appears to be a differential ER mRNA splicing between the *in vitro* and *in vivo* milieu.

Introduction

The estrogen receptor (ER*) is a ligand-inducible transcription factor which regulates the expression of a variety of genes including some growth factors, hormones and oncogenes important for the growth of breast cancer (1,2). Expression of the ER plays an important role in the pathogenesis and maintenance of breast cancer. In breast cancer patients, about two-thirds of tumors are ER-positive (3); 50% of these ER-positive tumors are estrogen-dependent and respond to endocrine therapy (4). Breast carcinomas occurring in postmenopausal women are often ER-positive (5), and many of

these tumors express significantly more ER than does the normal mammary epithelium (6).

On the other hand, it has been proposed that the cumulative exposure of breast tissue to hormones associated with ovarian activity is a major determinant of breast cancer risk (7,8). Furthermore, some environmental agents and dietary components might influence breast cancer development by functioning as xenoestrogens or estrogenic potentiating factors (9).

The ER gene spans 140 kb and is comprised of 8 exons which are spliced to yield a 6.3 kb mRNA, encoding a 595-amino acid protein with a molecular weight of 66 kd (10,11). The ER protein is comprised of several discrete functional domains (12). Two transcriptional activation functions (TAF-1 and TAF-2) reside in exons 1 and 8, respectively (13,14). The DNA binding domain of ~70 amino acids is located at exons 2 and 3. Exons 4 through 8 translating into 250 amino acids in size are necessary and sufficient for ligand binding (12,15). A 22 amino acid sequence necessary for subunit dimerization has been located in exon 7 (16).

Recently, it has been reported that both the wild type and variant estrogen receptors were coexpressed in some human breast carcinoma cell lines (17-23). Variant human breast tumor ER with constitutive transcriptional activity has been identified in tumor specimens (24,25). Therefore, estrogen receptor variants might have a role in human breast cancer. However, to date, the role of the estrogen receptor variant in carcinogenesis and the regulation of its expression are poorly understood.

The human mammary gland contains a small but distinct population of ER-positive cells, comprising ~7% of the total epithelial cell population from all biopsies (26). The ER-positive cells were distributed as scattered single cells, most of them (87%) were luminal epithelial cells or occupied an intermediate position in the duct wall. The highest frequency of ER-positive cells has been found in the lobules as compared to the interlobular ducts (26). Normal HBEC grown in the commonly used media, MCDB 170 (27) and DFCI-1 (28), including the commercially available normal HBEC (Clonetics), exhibited basal epithelial, but not luminal epithelial cell characteristics. These cells have not been shown to express the estrogen receptor.

Recently, we have developed a culture method to grow two morphologically and antigenically distinguishable normal human breast epithelial cell (HBEC) types from reduction mammoplasty (29). Type I HBEC is deficient in gap junctional intercellular communication and has luminal and stem cell characteristics, i.e. the differentiation of Type I into Type II HBEC by a cyclic AMP-inducing agent (29) and the unique ability of Type I HBEC to form budding and ductal structures on Matrigel matrix (30), whereas Type II HBEC is capable of gap junctional intercellular communication (29,31,32) and expresses basal epithelial cell phenotypes (29).

In this study, we characterized the expression of estrogen receptors in these two types of normal HBEC and the neoplastically transformed cell lines derived from these cells.

*Abbreviations: ER, estrogen receptor; ERE, estrogen responsive element; HBEC, human breast epithelial cell; kd, kilodalton; PBS, phosphate buffered saline; NGS, normal goat serum; PgR, progesterone receptor; RT-PCR, reverse transcription-polymerase chain reaction; SV40, simian virus 40.

Materials and methods

Cells and cell culture

Normal human breast epithelial cells were isolated from reduction mammaplasty of four different women (21–29 years of age, designated HME-5, -12, -14, -15). The media and the procedure used to develop the two types of normal HBEC have been described previously (29). The primary cultures developed *in vitro* for one week were stored in liquid nitrogen. Early passage cells, after recovery from liquid nitrogen storage, were used in these experiments. All cell cultures were grown at 37°C in incubators supplied with 5% CO₂ and humidified air.

Transformation of normal HBEC was achieved by lipofectin-mediated transfection of HBEC (29) with SV40 DNA (GIBCO-BRL) (M11SV2, M13SV1, M13SV22 derived from normal HBEC cultures HME-11 and HME-13, respectively) or a plasmid carrying the G418-resistance gene and an origin-defective SV40 genome expressing a wild type large T-antigen (pRNS-1 obtained from John S. Rhim of the National Cancer Institute) (M15SV1-11, M15SV21-29 derived from HME-15). In addition to the ER-positive MCF-7, T47D and the ER-negative MDA-MB-231 breast cancer cell lines, a HBEC line (M12B4 derived from HME-12), with extended lifespan after 5-bromodeoxyuridine treatment, was also included in the study of ER expression.

Immunofluorescence staining of estrogen receptor

Cells were fixed by 4% paraformaldehyde for 20 min, postfixed with absolute methanol for 30 s and then rehydrated with phosphate buffered saline (PBS). Subsequently, nonspecific binding sites were blocked with 10% normal goat serum (NGS) in PBS for 30 min at room temperature. The cells were incubated overnight at 4°C with a primary antibody against ER (Ab-1, Oncogene Science, NY; diluted 1:200 in PBS containing 0.1% bovine serum albumin and 1% NGS), washed three times with PBS, and then incubated with a second anti-mouse antibody conjugated with rhodamine (Jackson Immuno-research Lab, West Grove, PA; diluted 1:100 in PBS) for 30 min at room temperature. The cells were washed extensively with PBS containing 0.1% bovine serum albumin and 1% NGS and mounted with coverslips on Poly-aquamount (Polysciences, Inc., Warrington, PA). The cells were examined and photographed using the Ultima laser confocal scanning microscope (Meridian Instruments, Okemos, MI).

SDS-PAGE and Western blot analysis

Proteins were extracted from normal and SV40-transformed HBEC, and from MCF-7, T47D, MDA-MB-231 cells in 100 mm dishes by treatment with 20% SDS lysis solution containing several protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μ M leupeptin, 1 μ M antipain, 0.1 μ M aprotinin, 0.1 μ M sodium orthovanadate, 5 mM sodium fluoride). After sonication at three 10-s pulses from a probe sonicator, the cell lysates were stored at -20°C until use (31). The protein amounts were determined by the DC protein assay kit (Bio-Rad Co., Richmond, CA). Proteins were separated on 12.5% SDS polyacrylamide gels and transferred to PVDF membranes at 20 V for 16 h. ER was detected by the anti-ER monoclonal antibody (NCL-ER-LH2, Vector Lab., Burlingame, CA) which recognizes the amino (N)-terminal portion of ER and by the anti-ER monoclonal antibody (Ab-1, oncogene science, NY) which recognizes the carboxyl (C)-terminal portion of ER after blocking with 5% dried skim milk in PBS containing 0.1% Tween 20. This was then followed by incubation with horseradish peroxidase-conjugated secondary antibody and detected with the ECL chemiluminescent detection reagent (Amersham Co., Arlington Heights, IL). The membranes were exposed to X-ray film for 15 s to 1 min.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells by using Trizol reagent (GIBCO-BRL, Gaithersburg, MD) according to the manufacturer's protocol. The extracted RNA was dissolved in RNase-free water, and its concentration and purity was determined by a spectrophotometer.

cDNA was synthesized from the isolated RNA by reverse transcription in 20 μ l reaction solution containing 2.5 μ M of random hexamers (Perkin Elmer, Madison, WI), 50 units of Moloney murine leukemia virus reverse transcriptase (Perkin Elmer, Madison, WI), 1 μ g of total RNA, 2 μ l of 10 \times PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3), 5 mM MgCl₂, 1 mM of each dNTP, 20 units of RNase inhibitor (Perkin Elmer, Madison, WI), and 2 μ l of RNase free water (Promega, Madison, WI). The reaction mixture was incubated at room temperature for 10 min and then at 42°C for 15 min, heated to 99°C for 5 min, and then quick-chilled on ice. The 20 μ l of solution which contains the reverse transcribed cDNA were added to 30 μ l of PCR reaction mixture containing 2 mM MgCl₂, 3 μ l of 10 \times PCR buffer, 25 pmol of each primer and 1.25 units of AmpliTaq DNA polymerase (Perkin Elmer, Madison, WI). The primers chosen for PCR are sequences surrounding the border between exons 7 and 8 of the ER (5'-GCACCTGAAGTCTCTGGAA-3', 5'-TGGCT-

AAAGTGGTGCATGAT-3') (33) and sequences encompassing exon 2 of the ER (5'-TACTGCATCAGATCCAAGGG-3', 5'-ATCAATGGTGCACCTGGT-TGG-3') (34). The primers used to generate the 306-base pairs of GAPDH products are 5'-CGGAGTCAACGGATTGGTCTGAT-3' and 5'-AGCCTTC-TCATGGTGGTGAAGAC-3'. Thermal cycling was performed in a Gene-AMP 9600 PCR system (Perkin Elmer, Madison, WI) by using the following two steps amplification profile: an initial denaturation at 95°C for 105 s, 35 cycles of denaturation at 95°C for 15 s, and annealing at 60°C for 30 s; and a final elongation step at 72°C for 7 min. The PCR products were then electrophoresed in a 2% agarose gel and stained with 0.5 μ g/ml ethidium bromide. The size of cDNA product was determined by comparison to DNA size markers (the HaeIII digested X174 DNA, GIBCO-BRL, Gaithersburg, MD).

ER-ERE binding assay

Nuclear extracts were prepared as previously described (35). Cells were lysed with hypotonic buffer (10 mM HEPES, 1.5 mM, MgCl₂, pH 7.5) and nuclei were pelleted by centrifugation at 3000 g for 5 min. Nuclear lysis was performed using a hypertonic buffer (30 mM HEPES, 1.5 mM MgCl₂, 450 mM KCl, 0.3 mM EDTA, and 10% glycerol with 1 mM DTT, 1 mM PMSF, and 1 μ g/ml of aprotinin and leupeptin). Following lysis, the samples were centrifuged at 12 000 g for 30 min, and the supernatant was collected for use in the DNA binding assay. Double-stranded oligonucleotides, containing a wild-type estrogen responsive element (ERE) consensus sequence (5'-GTCCAAAGTCAGGTCAAGTACCTGATCAAAGTT-3'), that corresponds to -308/-342 of the promoter/upstream element of the *Xenopus* vitellogenin A2 gene (35), was synthesized at the Biotechnology Facility of Michigan State University. The ERE was annealed, and end-labeled with [γ -³²P]. The nuclear extract (3 μ g) was incubated with the reaction buffer [70 mM KCl, 30 mM HEPES (pH 8.0), 1.5 mM MgCl₂, 0.3 mM EDTA, 10% glycerol, and 2 μ g poly (di-dC)] in the presence or absence of unlabelled oligonucleotide competitor, or antibody for 10 min, followed by a 20 min incubation at room temperature with the ³²P-labelled ERE DNA probe. The products of ER and ERE DNA binding activity was separated from free probe in a 4.8% polyacrylamide gel by electrophoresis using TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA). After electrophoresis, the gel was dried and subjected to autoradiography.

Tumor development in athymic nude mice

Cells suspended in 0.25 ml of MSU-1 medium (29) with 5% fetal bovine serum were injected subcutaneously into each of two sites on a female athymic nude mouse (Harlan-Sprague-Dawley). The tumors formed from 6 \times 10⁶ cells inoculated in each site were dissected and measured four weeks after inoculation. The tumor cells were removed for protein extraction for Western blot analysis and for re-establishment of cell cultures which were also harvested for Western blot analysis.

Results

Expression of estrogen receptor in normal HBEC

Type I and Type II normal HBEC derived from reduction mammaplasty of four different women (HME-5, -12, -14, -15) were examined for their ER expression by Western blot analysis using an anti-ER antibody which recognizes the C-terminal region of ER (Ab-1, Oncogene Science). The results show that all the four Type I HBEC expressed an ER, whereas all the four Type II HBEC did not express any ER (Figure 1A and B). The ER expressed in Type I HBEC, however, is not the wild type ER since its molecular size (~48 kd) is smaller than the wild type ER (~66 kd). This variant ER appears to contain a deletion in N-terminal region, based on the observation that, unlike the ER-positive MCF-7 and T47D cells which express both wild type and variant ER, the ER was not detectable in Type I or Type II HBEC when anti-ER antibodies which recognize the N-terminal region (NCL-ER-LH2, Vector Laboratory; C314, Santa Cruz) were used (Figure 1C).

The expression of ER in Type I, but not Type II, normal HBEC was confirmed by immunofluorescence staining using Ab-1 anti-ER antibody (Figure 2C and E). In this study, the ER was found to be expressed in all Type I HBEC colonies examined (>100) and in every cell in a colony (data not shown). Similar to Western blot analysis, the ER was detectable

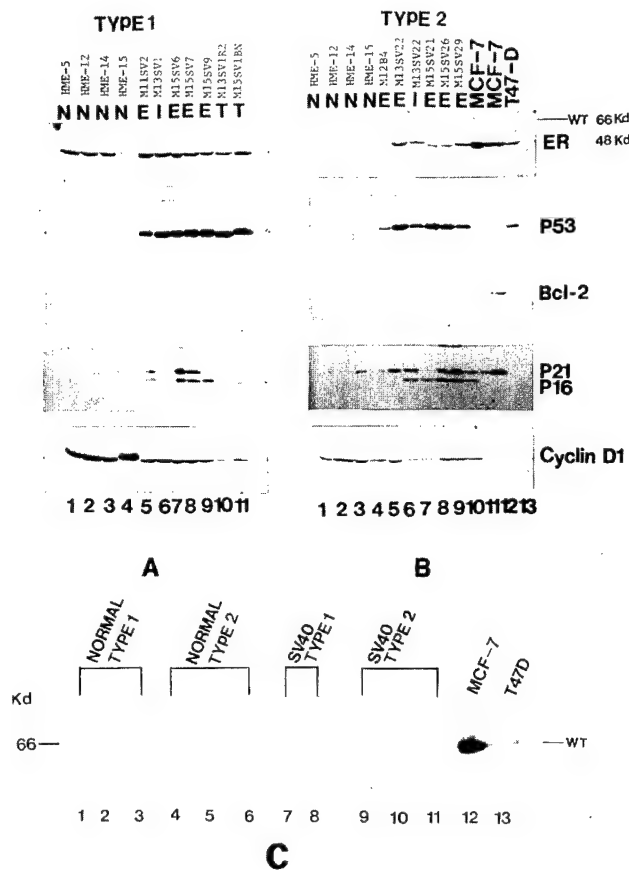


Fig. 1. Western blot analysis for the expression of estrogen receptor, p53, bcl-2, p21, p16, and cyclin D1 expression. (A) Same amount of protein from normal Type I (N) and SV40-transformed Type I HBEC at different stages of transformation (extended lifespan, E, immortal, I and tumorigenic, T) blotted on the same membrane were probed for the expression of cell cycle-related proteins and ER; (B) same as panel A, except normal Type II and SV40-transformed Type II HBEC and the control MCF-7 and T47D cells were used. In both experiments, the Ab-1 (Oncogene Science) anti-ER antibody which recognizes the C-terminal domain of ER was used; (C) the expression of ER in normal and SV40-transformed Type I and Type II HBEC was studied using the NCL-ER-LH2 (Vector lab.) anti-ER antibody which recognizes the N-terminal domain of ER. The cells used are HME-12, -14, -15 (lanes 1-3), HME-12, -14, -15 (lanes 4-6), M13SV1 (lane 7), M13SV1 (lane 8), M13SV22 (lane 9), M15SV26 (lane 10), M15SV29 (lane 11).

using the C-terminal anti-ER antibody (i.e. Ab-1) and not detectable using the N-terminal anti-ER antibodies (i.e. NCL-ER-LH2 and C314).

ER expression in SV40-transformed Type I and Type II HBEC

Similar to the Type I normal HBEC, SV40 transformed Type I HBEC lines (derived from three different primary cultures) at different stage of neoplastic transformation (extended lifespan, immortal, and tumorigenic) expressed the variant ER (~48 kd) (Figure 1A) at similar expression levels as their parental cells, detected by Western blotting. Interestingly, unlike Type II normal HBEC, SV40 transformed Type II HBEC lines also expressed this variant ER (Figure 1B). In contrast, a cell line with extended lifespan after 5-bromodeoxyuridine treatment (BrdU) (M12B4) did not express the ER (Figure 1B). As expected, the ER of SV40-transformed Type I and Type II HBEC were found in the nuclei (Figure 2D and 2F) similar to MCF-7 cells (Figure 2A) using the Ab-1 anti-ER antibody. However, we could not detect any ER in the SV40-transformed

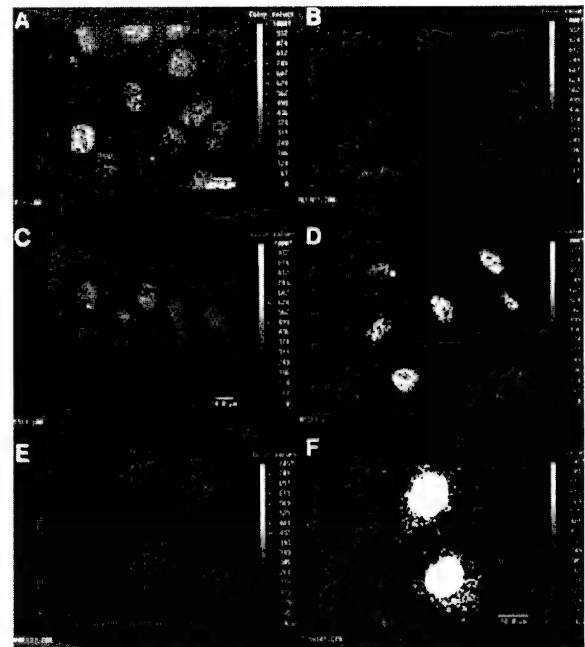


Fig. 2. Immunofluorescence staining for ER using the Ab-1 (Oncogene Science) anti-ER antibody. (A) and (B) MCF-7 with and without primary antibody, respectively as controls; (C) and (D) normal and SV40-transformed Type I HBEC (HME15 and M15SV1), respectively; (E) and (F) normal and SV40-transformed Type II HBEC (HME15 and M15SV29), respectively.

Type I and Type II HBEC, using antibodies which recognize the N-terminal region of the ER (NCL-ER-LH2, Vector lab; C314, Santa Cruz), by Western blot analysis (Figure 1C) or by immunofluorescence staining (data not shown). In order to understand the function of this variant ER, we also examined progesterone receptor (PgR) expression which might be induced by estrogen in cell strains and cell lines studied in Figure 1A and 1B. None of these cells were found to express the PgR (data not shown).

ER expression by RT-PCR analysis

To verify the above results and to gain more insight into the nature of the expressed variant ER, RT-PCR was performed using primer pairs encompassing exon 2 or a contiguous region bordering exons 7 and 8. As controls, RNA from the ER-positive MCF-7, T47D and the ER-negative MDA-MB-231 breast cancer cell lines were reverse transcribed, and then the cDNAs were amplified by PCR in conjunction with studies using Type I and Type II normal HBEC, as well as SV40-transformed Type I and Type II HBEC. As shown in Figure 3, when primer pairs surrounding a region bordering exon 7 and exon 8 of the ER were used for PCR, a ~470 base pair product was detected in Type I but not in Type II normal HBEC or in the ER negative MDA-MB-231 cells. The amplified DNA was also found in SV40 transformed Type I and Type II HBEC, as well as in the ER-positive MCF-7 and T47D cells. The RT-PCR condition appears similar for all the cell strains or cell lines tested as judged from the uniform control amplification for the GAPDH gene. When primer pairs encompassing the exon 2 of ER were used for the PCR, none of the normal or SV40 transformed Type I or Type II HBEC yielded the ~650 base pair product as did the ER-positive MCF-7 and T47D cells (Figure 4). These results confirm previous results that showed a variant ER was expressed in normal Type I HBEC and both Type I and Type II SV40 transformed HBEC.

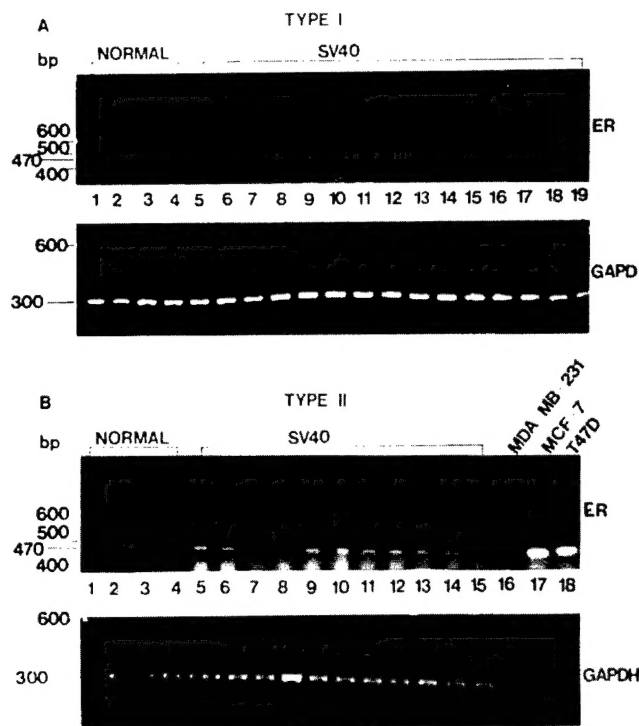


Fig. 3. RT-PCR analyses for detecting ER transcripts using primer pairs in the border between exon 7 and exon 8. (A) Type I and SV40-transformed HBEC lines: lanes 1–4 (HME-5, HME-12, HME-14, HME-15, respectively), lanes 5–19 (M11SV1, M11SV2, M13SV1, M13SV2, M15SV1–11, respectively); (B) normal Type II and SV40-transformed Type II HBEC lines: lanes 1–4 (HME-5, HME-12, HME-14, HME-15, respectively), lanes 5–15 (M13SV22, M15SV21–30, respectively). The control RT-PCR for GAPDH are also shown.

In addition, the study further indicates that the variant ER contains a deletion in the exon 2 region of the ER.

The ability of the variant ER to bind an estrogen responsive element

If the expressed variant ER contains a deletion in the DNA binding domain (exons 2 and 3), this ER is expected to lose its ERE DNA binding ability. To test if this is the case, nuclear extracts were prepared from cell lines expressing the variant ER and then subjected to a ER-ERE binding assay using a ERE motif, the cognate binding site for estrogen receptor. The results showed that the variant ER expressed in normal and SV40 transformed cells was deficient in ERE binding activity, while MCF-7 cells, as a positive control, produced a strong binding signal (Figure 5A). The specificity of the retarded ER-ERE band observed for MCF-7 cells was confirmed by the addition of an excess of unlabelled double-stranded oligonucleotide. As shown in Figure 5B, the retarded band was supershifted when the nuclear extract was preincubated with anti-ER antibody (Ab-1) before its binding to the ERE probe (Figure 5B). These results are consistent with previous RT-PCR and Western blot analyses and clearly indicate that this variant ER had a deleted DNA binding region.

Expression of wild type and variant ER in tumors formed in nude mice by neoplastically transformed Type I HBEC

The SV40 immortalized Type I HBEC line (M13SV1) reported previously (29) was non-tumorigenic. After X-ray irradiation (2 Gy, twice), large colonies formed in soft agar were found to be weakly tumorigenic in athymic nude mice (a cell line,

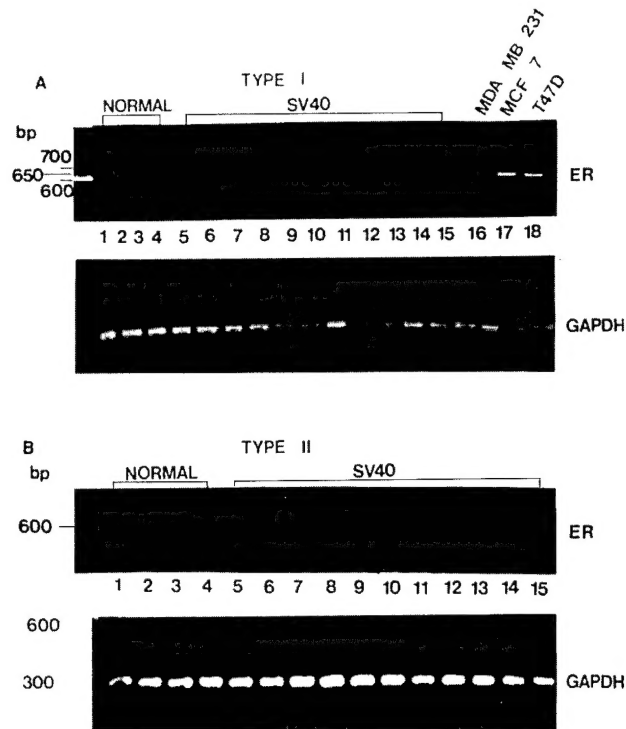


Fig. 4. RT-PCR analyses for detecting ER transcripts using primer pairs encompassing exon 2. (A) Type I and SV40-transformed HBEC lines: lanes 1–4 (HME-5, HME-12, HME-14, HME-15, respectively), lanes 5–16 (M11SV1, M13SV1, M15SV1–10, respectively); (B) normal Type II and SV40-transformed Type II HBEC lines: lanes 1–4 (HME-5, HME-12, HME-14, HME-15, respectively), lanes 5–15 (M13SV22, M15SV21–30, respectively). The control RT-PCR for GAPDH are also shown.

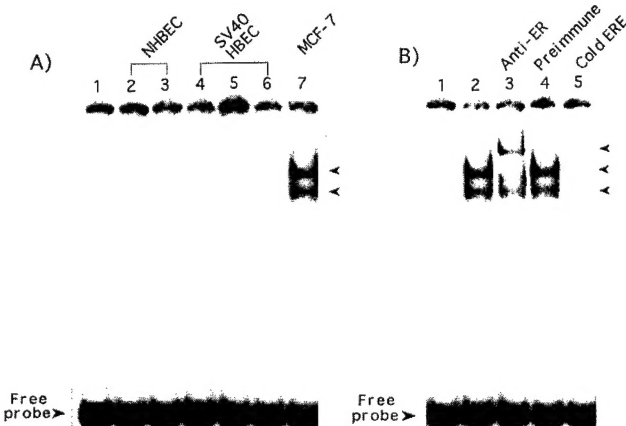


Fig. 5. Electrophoretic Mobility Shift Assay (EMSA). (A) ER-ERE binding: free probe only (lane 1), and nuclear extracts from normal Type I HBEC (lane 2, HME-15) and normal Type II HBEC (lane 3, HME-15), SV40-transformed Type I HBEC (lanes 4 and 5, M13SV1 and M15SV6, respectively), SV40 transformed Type II HBEC (lane 6, M13SV22) and MCF-7 (as a positive control, lane 7); (B) the specificity of the retarded bands was confirmed by the addition of an excess of unlabelled double-stranded oligonucleotide. The retarded band was supershifted dramatically when the nuclear extract was preincubated with anti-ER antibody (Ab-1, Oncogene Science) before binding to ERE probe (lane 3), lane 1, free probe; lane 2, MCF-7 without anti-ER antibody; lane 4, MCF-7 with preimmune serum from mouse; lane 5, unlabeled oligonucleotide of ERE.

M13SVIR2 was used in this study). The weakly tumorigenic cells became highly tumorigenic (tumor > 1 cm formed in 4 weeks) after infection with a viral vector carrying a mutated *neu* oncogene (i.e. M13SV1R2-N1, -N4, -N8) (manuscript in

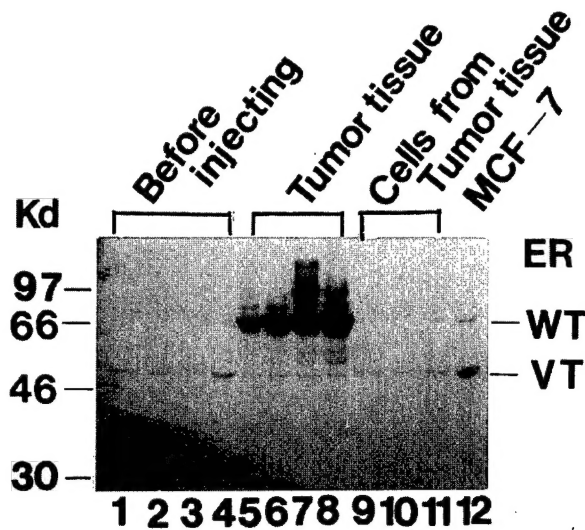


Fig. 6. Western blot analysis for the expression of ER. The expression of ER in weakly tumorigenic cell line, M13SV1R2 and highly tumorigenic cell lines, M13SV1R2-N1, -4, -8, before inoculation for tumor growth in nude mice (lanes 1–4) and after reestablishment of cell culture from tumors (lanes 9–11), were compared with that in tumors formed by these cells (lanes 5–8). The cell lysates loaded are: lanes 1, 5, 9, M13SV1R2; lanes 2, 6, 10, M13SV1R2-N1; lanes 3, 7, 11, M13SV1R2-N4; lanes 4, 8, M13SV1R2-N8. The positions of wild type ER (WT) and variant ER (VT) are indicated.

preparation). We have tested the expression of ER in these tumorigenic cell lines before inoculation in nude mice, in tumors formed by these cells in nude mice and in cell cultures reestablished from tumors. The results showed that cells, before injection into nude mice, and cells, reestablished from tumor tissue, *in vitro*, only expressed the variant ER (~48 kD) using an anti-ER antibody recognizing the C-terminal region of the ER (Ab-1, Oncogene Science). Significantly, the tumor tissues expressed a high level of the wild type ER (~66 kD) and less amount of the variant ER (~48 kD) by Western blotting using the Ab-1 (Oncogene Science) (Figure 6) or the human ER specific antibody (D75, kindly provided by Dr Geoffrey Greene of the University of Chicago) (data not shown). Western blot analysis for the expression of progesterone receptor using an anti-PgR antibody (Ab-1, Oncogene Science) was also carried out in these cells. The results are similar to that observed for the expression of ER (i.e. expression was found in tumor tissues but not in cells grown *in vitro*) (Figure 7), indicating that the wild type ER expressed *in vivo* might be functional.

Expression of cell cycle related proteins (*p53*, *p21^{waf}*, *p16^{INK4}*, *cyclin D1*)

The same Western blots studying ER expression (Figure 1) were also probed for the expression of cell cycle related proteins. As expected, high levels of *p53* were found in SV40 transformed cell lines, confirming the involvement of large T antigen in their transformation. The *p21* and *p16* proteins were frequently elevated in transformed Type I and Type II HBEC cells. The cyclin D1 was highly expressed in Type I normal HBEC compared to their Type II HBEC counterparts. The transformation by SV40 reduced the expression of cyclin D1 in Type I but not Type II HBEC.

Discussion

The major finding of this study is that an estrogen receptor was expressed in all the Type I HBEC with luminal and

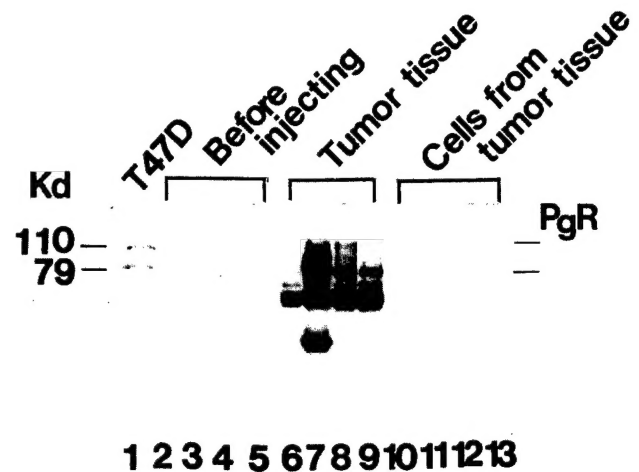


Fig. 7. The proteins from cell lines used in experiments presented in Fig. 6 also were used for Western blot analysis for the expression of PgR. The PgR expression in these cells before inoculation for tumor growth in nude mice (lanes 2–5) and after re-establishment of cell culture from tumors (lanes 10–12) were compared with that in tumors formed by these cells (lanes 6–9). The cell lysate loaded are: lanes 2, 6, 10, M13SV1R2; lanes 3, 7, 11, M13SV1R2-N1; lanes 4, 8, 12, M13SV1R2-N4; lanes 5, 9, 13, M13SV1R2-N8.

stem cell characteristics examined while their Type II HBEC counterparts with basal cell characteristics did not express any ER. Furthermore, all the SV40 transformed Type I and Type II HBEC lines examined (15 and 11 cell lines, respectively) were also found to express the ER. Although the human mammary gland is known to contain a small population of ER-positive cell (26), the previously reported normal HBEC in culture have not been shown to express significant level of ER. The expression of ER in our Type I HBEC or SV40 transformed cells is unambiguous since it has been observed by three different methods (i.e. Western blot analysis, immunofluorescence staining, and RT-PCR).

The ER expression, however, is not the wild type ER. It is a variant ER with smaller molecular weight (~48 kD) than the wild type ER (~66 kD). This variant ER was detectable by Western blot analysis and immunofluorescence staining using an anti-ER antibody (Ab-1, Oncogene Science) recognizing the C-terminal portion of the ER but was undetectable when anti-ER antibodies recognizing the N-terminal portion of the ER (NCL-ER-LH2, Vector laboratory, C314, Santa Cruz) were used. This observation suggests that the variant ER contains a deletion in the N-terminal region. By RT-PCR analysis using primer pairs in the C-terminal or N-terminal region, the deletion was found to be in the exon 2 region. Since exon 2 is a part of DNA binding domain, the ER with deletion in this region is expected to lose its DNA-binding activity. This was found to be true in the ER-ERE binding assay. That the variant ER deleting DNA binding domain would be non-functional is revealed by the non-expression of the PgR which is positively regulated by the ER (36). Recently, ER variants with deletion in exon 2, exon 3, or both were observed in normal human breast tissue (37). Further RT-PCR analysis will reveal whether our variant ER also includes deletion in exon 3.

Of the eight exons that constitute the ER mRNA transcript, most of them (exons 2–7) have been found to be involved in aberrant splicing events in breast tumor cell lines or tissues (38–42). Many of these variants disrupt critical regions such that they become non-functional (20). The variant ER expressed

in our cells appears to belong to this category. Some other variants may play an important role in tumor growth, e.g. variant ER lacking the hormonal binding domain is suspected to be a dominant positive transcriptional activator that accounts for hormone independent growth of tumors (24,25). Furthermore, variants ER lacking the DNA binding domain might function as negative or positive transcription factors through protein-protein interaction (43-45). Indeed, Yang *et al.* have recently shown that human transforming growth factor- β 3 gene can be activated by the estrogen receptor in the presence of estrogen metabolites or estrogen antagonists. Activation was mediated by a polypurine sequence, termed the raloxifene response element, and did not require the DNA binding domain of the estrogen receptor (45).

Although the ER expressed in Type I and SV40 transformed HBEC appears to be non-functional, there is evidence that this is only an *in vitro* phenomenon. From studies presented in Figures 6 and 7, tumorigenic cell lines derived from Type I HBEC were found to express high level of wild type ER in tumors formed by these cells in athymic nude mice. The same cells did not express the wild type ER before inoculation for tumor growth or after the tumor cells were cultured *in vitro*. In this study, the wild type ER expressed in tumors have been shown to be human ER as they were observed by Western blot analysis using a human ER specific antibody. The expressed wild type ER in tumors appears to be functional since the PgR was simultaneously expressed. The differential *in vitro* and *in vivo* expression of different splicing variants of ER could be due to factors provided only by the *in vivo* condition such as 3-dimensional tissue structure or the presence of stromal cells and extracellular matrix. This will be examined in future studies.

The expression of the variant ER in SV40 transformed Type II HBEC is unexpected. The mechanism for its expression is not clear. One possible mechanism is that the expression of large T-antigen induced the expression of ER. We have tested the hypothesis by transfecting the ER-negative MDA-MB-231 breast adenoma cells with SV40. The MDA-MB-231 cells expressing the large T-antigen resulted from SV40 transfection, however, remained ER-negative (our unpublished results). Alternatively, in the Type II HBEC population, there might exist a small population of ER-positive transitional cells, newly differentiated from Type I cells, which were the target cells for SV40 transformation. Except for ER-expression, the phenotypes of SV40 transformed Type II HBEC are substantially different from that of SV40 transformed Type I cells (29). Therefore, these hypothetical transitional cells are quite different from the Type I HBEC.

The implication of the results from this study are two-fold. First, the expression of ER in Type I HBEC provides additional similarity between breast cancer cells and Type I HBEC. As mentioned previously, the origin of the ER-positive tumors is not known. One possible origin is that they were derived from ER-positive normal HBEC stem cells similar to our Type I HBEC. During the neoplastic transformation, many of the parental target cell phenotypes are largely preserved. Those include deficiency in gap junctional intercellular communication, expression of luminal epithelial cell markers (29), and expression of telomerase (our unpublished results). Our study also indicates that Type I HBEC are more susceptible to neoplastic transformation by an oncogenic (SV40) stimulus (i.e. to become immortal and capable of anchorage independent growth) (29). The phenotypic similarity between Type I HBEC

and breast cancer cells mentioned above are consistent with the notion of oncogeny as blocked or partially blocked ontogeny (46). Second, our results indicate that there is a differential splicing in ER mRNA under *in vitro* and *in vivo* conditions. The expression of a variant ER under our *in vitro* culture condition might be an indication that the same cells might express the wild type ER *in vivo*. Furthermore, the results suggest a potential strategy to control the growth of estrogen-dependent human breast tumors, i.e. the modulation of the tissue environment in order to facilitate the alternative splicing that results in the expression of non-functional ER. Lastly, this study raises an awareness concerning limitations in interpretation of experimental results. First, the use of immunostaining to classify tumors as ER-positive or ER-negative based on the use of one antibody may not be adequate. It may not detect all alternatively spliced ER nor provide the information concerning its function. Second, the phenotype of a normal cell type used for comparison with that of tumor cells may be irrelevant. For example, when we describe normal HBEC as proficient in gap junctional intercellular communication and ER-negative, they apply to Type II but not Type I HBEC. In other words, not all normal cells have the same phenotype in a given tissue. Thirdly, gene expression *in vitro* could be dramatically different from gene expression *in vivo*. This is exemplified by the wild type ER expression in Type I HBEC-derived tumorigenic cells as demonstrated in this study.

Acknowledgements

We gratefully acknowledge the gift of the D75 antibody against human ER provided by Dr Geoffrey Greene. This work was supported by grants from the National Institute of Environmental Health Sciences (ES07256) and the National Cancer Institute (CA21104). The word processing skills provided by Robbyn Davenport are acknowledged.

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Received on August 19, 1996; revised on October 21, 1996; accepted on November 22, 1996